RESEARCH REPORT
PORK SAFETY

Title: Surface Material, Temperature, and Soil Effects on Pathogen Growth in Condensate - **NPB# 02-048**

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Abstract: In the meat processing environment, bacterial contamination of food contact and non-contact surfaces can lead to post-process contamination of product through direct or indirect transfer of organisms from these surfaces. Moisture is a requirement for survival. One source of moisture common in food factories is condensation, formed when moist air contacts cool surfaces. Condensate can carry nutrients within bacterial growth niches and can act as a carrier of pathogenic and spoilage organisms from these niches on to product. We evaluated four surfaces [stainless steel, acetal resin, fiberglass reinforced plastic wall paneling (FRP), and mortar] under a variety of conditions to determine the effects that combinations of surface type, soil, and temperature have on the survival of three pathogens (*Listeria monocytogenes*, *Salmonella*, and *Yersinia enterocolitica*), in the presence of condensate. Half of the surface coupons to be tested were soiled by dipping into sterile porcine serum and then allowed to dry. Coupons were exposed to cell suspensions made up of five-strain cocktails of each organism (inoculation with each organism was performed separately on separate coupons) of ca. 10^7 CFU/mL of Butterfield's phosphate buffer (BPB) and incubated for 2 hours at 25ºC to allow the cells to attach. Coupons were rinsed to remove unattached cells and then placed into sterile Petri dishes containing moistened filter paper disks to generate a 100 % relative humidity environment upon sealing of the dish. Coupons were then placed into either a 4ºC or 10ºC incubator and sampled at either six time intervals over a 15 day period (for stainless steel, acetal resin, and FRP) or ten intervals over 120 h (for mortar). Cells were removed from the surfaces of stainless steel, acetal resin, and FRP by vigorous shaking in 100 mL BPB with 3 g glass beads and from mortar by sonication in 100 mL BPB for 30 s. Cell populations, reported as CFU/coupon, were determined by plating onto Tryptic Soy Agar with 0.6 % Yeast Extract.

Mortar had the most lethality of any of the four surfaces studied with *Listeria* and *Salmonella* surviving better than *Yersinia*. Stainless steel did not support the survival of *Listeria* as well as acetal resin or FRP when compared across all soil and temperature conditions.

However, for *Salmonella* and *Yersinia*, acetal resin was less supportive than stainless steel, with FRP sustaining the highest populations (least reduction). Temperature had little effect on survival of all three organisms across all surfaces, except for *Yersinia* on FRP at 10ºC, which displayed growth as opposed to death at 4ºC. Pork serum had a protective effect on the survival of *L. monocytogenes* on all four surfaces tested, with populations sustained at significantly ($P \leq 0.05$) higher numbers over time than unsoiled coupons. The presence of serum did not have an effect on the survival of *Salmonella* or *Yersinia* on stainless steel, acetal resin, or FRP, yet showed a significant protective effect on mortar. These results illustrate the importance of effective and timely cleaning and sanitization of both product contact and non-contact surfaces in order to prevent prolonged survival or growth of pathogens and possible contamination of post-process product.

Introduction: Product contamination from the post-processing environment is the most frequent cause of product contamination-based recalls, food poisoning outbreaks, and shelf-life problems in North America (1). No factory can be expected to be free of microorganisms. Operating a sterile food-processing factory would be exceedingly costly and impractical, even if possible. Factory conditions that promote growth of microbes increase the risk of post processing product contamination. Many factors affect the growth of microorganisms including moisture, nutrients, pH, oxidation-reduction potential, temperature, presence or absence of inhibitors, interactions between microorganisms in a population, and time (2). Conditions exist for the growth of microorganisms in most food processing factories (2). Moisture is the most critical of these as it is absolutely required for microbial growth (2). Air, water, tools, workers, traffic, and other means transfer microbes in the non-sterile factory environment into niches that are inaccessible for cleaning and sanitation. Within these niches many bacteria can attach themselves to underlying surfaces using cell membrane-bound structures (proteins, polysaccharides, glycoproteins, etc.), given enough time. Furthermore, if the appropriate conditions exist (ample nutrients, water, and time), biofilms can develop consisting of many types of bacteria and fungi entrapped in a glycocalyx matrix which can shield these organisms from cleaners and sanitizers (3, 4). Attached cells have also been correlated with increased heat resistance (4).

Equipment and maintenance/repair practices that entrap moisture result in microbial growth niche development (2). The same is true for operating conditions that release moisture into the environment. The water activity in niches also impacts the type of microflora that develops therein (2). Disruption of these niches can result in direct or indirect contamination of the product stream (2). The probability of product contamination is affected by a number of variables including but not limited to a) proximity of microbial growth niches to the product stream, b) number of niches, c) spatial relationship of niches to product stream, d) microbial populations in niches, e) extent of niche disruption, and f) exposure of the product stream to the environment (2).

Condensation forms when cold surfaces contact warm, humid air. Entrapment of condensate in surface microenvironments (e.g. microscopic pores, irregularities) could result in niches with high microbial populations. Cold, moist niches will favor the growth of cold-tolerating microbes over that of others. Cold-tolerating microbes of public health significance include *Yersinia enterocolitica*, *Listeria monocytogenes*, and selected strains of *Salmonella spp*. (5, 6). The chemical and physical nature of these microenvironments and the degree to which they can bind or entrap food residue is likely to play an important role in the growth and/or survival of selected microorganisms. The four surfaces studied in this experiment were as follows: 1) stainless steel which is used widely in food processing environments, 2) Delrin® by DuPont, an acetal resin

used to make various machine parts (e.g. slicer parts, conveyor belt sprockets and rollers) which require low friction between surfaces and durability, 3) fiberglass reinforced plastic wall paneling (FRP) which is often used in coolers and other rooms where the walls undergo frequent cleaning and need to be impermeable to water, and 4) mortar, as used in concrete flooring and walls.

Objectives:

1. To determine the influence that selected food contact and non-food contact materials that may be used in the ready-to-eat pork production environment have on the survival of selected bacterial food pathogens, attached to these surfaces, in the presence of condensate.
2. To determine the impact of temperature on the survival of selected bacterial pathogens in the presence of condensate.
3. To determine the effect soiling selected surfaces has on the survival of selected food pathogens in the presence of condensate.

Materials and Methods:

Culture Preparation

The isolates used and their origin, if known, where as follows: *Listeria monocytogenes* serotypes 4b (beef), 4b (processed meat), 1/2a (meat processing environment), 3a (meat), and 1/2b (pork sausage); *Salmonella* serotypes Agona (cereal), Typhimurium DT104 (clinical), Heidelberg (swine), Enteriditis, and Anatum (cattle); and *Yersinia enterocolitica* isolate 7YP (undetermined serotype) and isolates from the CDC of serotypes 0:8 (Pasteur Institute Collection), 0:9 (Pasteur Institute Collection), 0:3 (environmental-swine farm), and 0:5,27 (clinical). Cultures were stored at -80º C on Cryobeads™ (TSC, Ltd., UK).

Starting with an isolated colony streaked from the frozen culture onto Tryptic Soy Agar with 0.6 % Yeast Extract (TSA-YE) (Beckton, Dickinson and Company, MD), all isolates were transferred three times (24h; 37ºC) in Trypticase Soy Broth with 0.6 % Yeast Extract (TSB-YE) (Beckton, Dickinson and Company, MD) before use in the experiment to ensure an active culture. On the final transfer, 100 µL of each isolate in TSB-YE was inoculated into 100 mL (30 mL for mortar experiments) of TSB-YE and incubated (24h; 37ºC). Cells where centrifuged (10 min. @ 5520 x g), after the final transfer, using a Beckman J2-MI centrifuge (Beckman Coulter, Inc., CA), washed three times and resuspended to 100 mL (30 mL for mortar experiments) with sterile Butterfield's phosphate buffer (BPB).

Preparation of Surface Coupons

Coupons of stainless steel (type 304; #4b finish), fiberglass reinforced plastic wall paneling (FRP), and Delrin® by DuPont, an acetal resin, where cut measuring 8 x 3.3 cm. Surfaces were wiped with acetone to remove any oil residue, hand-washed in hot water with Terg-A-Zyme (Alconox, Inc., NY), rinsed in deionized water, sonicated (FS30H Ultrasonic Cleaner, Fisher Scientific, PA) in an alkaline solution of 2% Microsoap (International Products Corporation, Burlington, NJ) at 60ºC for 5 minutes, and then rinsed again in deionized water. Coupons were autoclaved for 15 minutes at 121º before use.

Mortar samples were formulated, mixed, poured, cured, and cut (2.5 x 2.5 x 1.3 cm) under defined conditions at the direction of Dr. Kimberly Kurtis, Assistant Professor, School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta. The mortar formulation was based on mixes used in industrial flooring applications. See Table 1 for quantities of each ingredient. Mortars were prepared by first mixing ASTM

type I cement, Class C fly ash and natural siliceous sand, obtained from Brown Brothers Quarry in Junction City, Georgia, USA, in a Hobart mixer for 3 minutes at 60 rpm. Separately, superplasticizer (ADVA Flow superplasticizer, Grace Construction Products; 3.1 µL per gram of cement) was added via a pipette to deionized/distilled water. Subsequently to the 3 minutes of dry ingredient mixing, the water and superplasticizer were slowly added, to improve workability, to the mixer and mixed for another 5 minutes at 120 rpm from the first addition of water. From this mix, 2.5 x 28.8 x 1.3 cm beams were cast in brass molds. These molds were placed on a vibrating table for at least 5 minutes to aid compaction. After compaction, samples were allowed to set for 5 minutes before the addition of the surface hardener (Maximent HD, ChemRex, Shakopee, MN). The surface hardener was sprinkled (4.9 kg/m^2) on the exposed surface of the mold and a trowel was used to finish the surface. Samples were demolded after 24 hours and placed in a limewater curing tank for an additional 27 days at 20±2º C. Furthermore, the samples were cut with a diamond blade masonry saw to the desired 2.5 x 2.5 x 1.3 cm dimensions. Coupons were brushed by hand to remove excess mortar dust and autoclaved for 15 minutes at 121ºC before use.

Soiling of Coupons

All coupons to be soiled where dipped into sterile pork serum (Atlanta Biologicals, Atlanta, GA) and allowed to dry for at least 45 minutes before inoculation.

Inoculation of Coupons

Coupons were inoculated by immersion into cell suspensions of ca. 10^7 CFU/mL of BPB prepared from a five-strain cocktail made of each organism type evaluated (either *Listeria*, *Salmonella*, or *Yersinia*). Coupons were incubated at 25ºC for 2 hours to allow attachment of cells to the surfaces. Afterwards, the coupons were rinsed by dipping into consecutive buckets of sterile BPB [two for stainless steel, FRP, and acetal resin (8 L in each) and five for mortar (12 L in each)] to remove unattached or loosely attached cells. The unfinished side of FRP was wiped with 70 % ethanol to remove and kill attached cells. This was due to the fact that in a processing environment only the finished side would be exposed. [Note: CFU counts for the finished side (26.4 cm^2) were doubled to yield a count for a surface area totaling 52.8 cm^2. Counts on mortar were doubled as well, since the coupons had approximately 25 cm^2 of surface area.]

Incubation of Coupons

After rinsing, coupons were placed into sterile Petri dishes (150 mm diameter) (individual Petri dishes contained three coupons of one surface and organism-type, either soiled or non-soiled, to be held at a specific temperature) which contained sterile filter paper disks, moistened with ca. 3 mL sterile water, on the bottom along with sterile wooden or plastic supports to keep the coupons raised above the filter paper (Figure 1). The Petri dishes were then sealed using Parafilm M (Pechiney Plastic Packaging, WI) to produce a 100% humidity environment and incubated at either 4ºC or 10ºC*.

**Salmonella* was only incubated at 10ºC on stainless steel, acetal resin, and FRP due to the fact that most *Salmonella*, unlike *Listeria* and *Yersinia*, are not psychrotrophs and thus do not have growth potential at 4ºC. Furthermore, the results of our experiments show that temperature did not have a significant effect on survival on mortar (see Figure 3), therefore the decision was made that it was not necessary to evaluate survival at 4ºC on the other surfaces.

Sampling of Coupons

For stainless steel, FRP, and acetal resin, sampling took place immediately after attachment and rinsing (day 0) and then on days 3, 6, 9, 12, and 15. Three coupons of

each surface type per soiled condition and temperature were removed from incubation at each sampling time. Mortar coupons were sampled over ten time periods spanning 120 hours.

Sampling for stainless steel, FRP, and acetal resin consisted of removing coupons from the sealed Petri dishes and placing each coupon, on edge, into a separate jar containing 100 mL BPB plus 3 g glass beads (Sigma; 425-600 µm diameter). Each jar was shaken on an orbital shaker (New Brunswick Scientific, NJ) at 350 rpm for 2 minutes to remove attached cells. An aliquot of the buffer was then plated onto TSA-YE and incubated 24-48 hours at 37º C, after which colonies were enumerated.

Mortar coupons were sampled by placing them into jars containing 100 mL sterile BPB and sonicating them for 30 seconds, without heat, and then plating an aliquot of the buffer onto TSA-YE. For sampling periods ≥ 24 hours after inoculation, the remaining buffer in each jar was enriched by adding 15 mL 5X TSB-YE, incubated for 48 hours and streaked on TSA-YE. In instances where the TSA-YE plates had zero colonies but the enrichments had visible growth, a loopful (10 µL) of the enrichment media was streaked for isolation to selective media (MOX for *Listeria*, XLT4 for *Salmonella*, and *Yersinia* Selective Agar for *Yersinia*). The presence of typical colonies was considered as positive confirmation.

Experimental Design and Statistical Analysis

A split-plot design was used with temperature, soil, and surface-type as independent variables. Two to three coupons were sampled for every condition at each sampling time and entire experiments were replicated three times. Data were pooled from the three experimental replications and analyzed using a general linear model in SAS (Statistical Analysis Software, SAS Institute, Cary, NC). Mean separations were performed using Fisher's student *t*-test.

Results:

Objective 1: To determine the influence that selected food contact and non-food contact materials that may be used in the ready-to-eat pork production environment have on the survival of selected bacterial food pathogens, attached to these surfaces, in the presence of condensate.

Mortar had the most lethality of any of the four surfaces studied. As shown in Figure 4, *Yersinia* had more than a 5 log decrease in population in only a 24 hour period and could only be detected by enrichments out to 120 hours (Table 3). *Listeria* and *Salmonella* (Figures 2 and 3, respectively) had better survival on mortar than *Yersinia* throughout the 120 hour incubation period.

Stainless steel did not support the survival of *Listeria* as well as acetal resin or FRP when compared across all soil and temperature conditions (Table 2). However, for *Salmonella*, acetal resin and stainless steel were both less supportive than FRP which sustained the highest populations (least reduction). There were no significant differences ($P > 0.05$) between population changes for Yersinia on any of the three surfaces across all soil and temperature conditions.

Objective 2: To determine the impact of temperature on the survival of selected bacterial pathogens in the presence of condensate.

Temperature appears to have very little effect on the survival of *Listeria monocytogenes*, in general. The only significant differences ($P \leq 0.05$) seen between 4ºC and 10ºC on stainless steel were at day 6. The organisms also declined more rapidly at 10ºC than at 4ºC after 24 h on mortar (Figure 2).

Salmonella was only evaluated on mortar at both 4ºC and 10ºC (Figure 3). The other *Salmonella* inoculated surfaces were tested at 10ºC. Overall, there was no significant difference between the effects of the two temperatures on the survival of *Salmonella*. Only at 48 hours was there a significant difference ($P \leq 0.05$) with 10ºC having a 0.65 log higher average population.

Unlike *Listeria* and *Salmonella*, temperature had an effect on the survival of *Yersinia enterocolitica* on stainless steel, acetal resin, and FRP, with incubation at 10ºC sustaining the populations (and even allowing growth on FRP) more so than at 4ºC. This effect is the most profound at day 15 for each of these surfaces (Figure 4). On mortar, however, there was no overall difference in survival of *Yersinia* noted between the two temperatures, with the exception of one sampling time (16 h) (Figure 4).

Objective 3: To determine the effect soiling selected surfaces has on the survival of selected food pathogens in the presence of condensate.

As illustrated in Figure 2, serum had a protective effect on the survival of *L. monocytogenes* on all four surfaces tested, sustaining populations at significantly ($P \leq 0.05$) higher numbers over time than unsoiled coupons. The presence of serum did not have an overall significant effect on the survival of *Salmonella* or *Yersinia* on stainless steel, acetal resin, and FRP, yet showed a significant protective effect on mortar (Figures 3 & 4, respectively). However, the protective effect for *Salmonella* on mortar was much more pronounced than for *Yersinia*.

Additional observations

There were no significant differences ($P > 0.05$) among the mean CFU/coupon of *L. monocytogenes*, *Y. enterocolitica* or *Salmonella* upon initial attachment onto any of the four surfaces (unsoiled), with one exception. Attachment of *Listeria* to stainless steel was significantly less than on FRP as determined by Fisher's student *t*-test ($P \leq 0.05$).

Discussion: Bacterial survival while attached to a surface is dependant on many factors. Temperature, time, and the availability of nutrients and water to bacterial populations are all important. The attachment surface itself plays a role in how well an organism can survive. The physicochemical properties of a surface influence cellular as well as nutrient attachment. The chemical nature of a surface (surface charges, pH, hydrophobicity/hydrophilicity) can either inhibit or promote attachment and survival of different organisms. In most cases, bacteria attach more to hydrophilic (stainless steel and glass) than hydrophobic surfaces (Teflon, nylon, buna-N rubber and fluorinated polymers) (7, 8, 9).

Substratum preconditioning, or the absorbance of organic molecules and charged ions to a surface before bacterial attachment, can have varying effects on how well a cell is able to attach to and survive on that surface. Fletcher et. al (10) showed that bovine serum albumin inhibited bacterial attachment to various surfaces. This effect, though, could not only be a property of the conditioning layer but also be due to the bacterial surface being modified by the albumin.

If a surface is able to chelate ions and/or prevent microbial access to vital organic nutrients then the chances of bacterial survival on that surface, without replenishment of these materials (such as in an aqueous environment), would be low. Our initial hypothesis was that given a soiled surface and condensate there would not only be survival, but also growth, to some extent (as shown for *Y. enterocolitica* on soiled FRP). But, our results show relatively stable populations in some instances (e.g. *L. monocytogenes* on FRP with serum, *Yersinia* on stainless steel with serum) and declines in others (e.g. *Salmonella* on stainless steel with serum). Population death, in

the presence of serum, could be due to either insufficient amounts of required nutrients in the serum to support growth or the binding of these nutrients to the surfaces thus preventing their uptake into the cells. Given that porcine serum was the only soil evaluated, it is possible that other porcine tissues (muscle, bone, organ, etc.), especially in combination, could provide for not only survival, but growth (as is the case for *Yersinia* on soiled FRP). Further study could be done to answer these questions. The ability of these organisms to survive for at least fifteen days, even on unsoiled surfaces, demonstrates that effective cleaning and sanitization of such surfaces in a processing environment is critical.

The presence of highly alkaline or acidic components can inhibit survival of bacteria. Mortar has a pH of about 12.5 due to CaOH (11). *Yersinia enterocolitica* populations declined much faster on soiled mortar than did *Listeria* and *Salmonella*. However, the death rate for *Yersinia* and *Salmonella* were similar on unsoiled coupons. *Listeria* survival was greater on unsoiled mortar coupons than both *Yersinia* and *Salmonella*, suggesting that *Listeria* are more tolerant to alkaline conditions. Taormina and Beuchat (12) demonstrated that *L. monocytogenes* can survive at least 144 h (with a 4 log unit decrease) at pH 11. The protective effect of soil to the survival of all three organisms on mortar could be due to the buffering capacity of the serum which slowed the damage to the cells by the high pH. The observation that cells (especially *L. monocytogenes* and *Salmonella*) are able to survive, with and without soil, on mortar for at least 120 hours, illustrates the need for proper cleaning and sanitization of walls and floors made from cement. However, the impact on bacterial attachment, survival or growth of various surface coatings and hardeners is unknown.

Lay Interpretation: Understanding how pathogenic bacteria such as *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Salmonella* can survive on surfaces commonly found in a meat processing environment is important for several reasons. It is necessary to gain knowledge of what is necessary for survival, where in the processing environment survival is most encouraged (walls, floors, machinery, etc.) and what the population dynamics of the organisms in these environments are. Does a bacterial population increase or decrease in numbers or will it remain constant? How long will it survive or how quickly will it increase? This information can help processors in determining how often to clean and sanitize and which surfaces and conditions might support bacterial survival more than others. It can also assist with respect to selection of appropriate surfaces when factory or processing equipment construction or renovation is being considered.

The results of our study show that on the four surfaces studied (stainless steel, acetal resin, fiberglass reinforced plastic wall paneling, and mortar), populations of the bacteria that were tested tended to either remain fairly constant or decline over the test period. The presence of pork serum provided increased survival, but not necessarily growth, over time. The temperature at which the surfaces and bacteria were incubated did not have much effect on the survival of the bacteria, with the exception that *Yersinia enterocolitica* was able to grow slowly on FRP at 10ºC over 15 days, but declined in numbers at 4ºC. Mortar was the most lethal of the four surfaces, most likely due to its high alkalinity (pH of about 12.5).

In conclusion, our study reveals that pathogens can survive for many days, even without the presence of nutrients and at refrigeration temperatures, on various surfaces. Therefore, prudent control over the processing environment through careful design and routine cleaning and sanitation are of vital importance in the prevention of contamination of product.

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Appendix:

Table 1. Cement mortar mix design per liter

Type I portland cement	573.2 g
Class C fly ash	172.0 g
Sand (FM=1.80)	1117.7 g
Deionized/distilled water	334.9 g
ADVA Flow superplasticizer	1775 µL

Table 2. Change in populations over 15 day period*

Surface	*Listeria monocytogenes*	*Salmonella*	*Yersinia enterocolitica*
Stainless steel	-3.39 B	-2.60 B	-0.91 A
Acetal resin	-1.85 A	-3.10 B	-1.43 A
FRP	-1.79 A	-1.02 A	-0.28 A

* Values (given in $\log_{10}$ cfu/coupon) are derived from overall data for all soil and temperature conditions. Significant differences ($P \leq 0.05$) between population changes among surfaces for each organism are designated by different letters following the numerical value.

Table 3. Recovery of organisms on mortar by enrichment

Bacteria	Conditions		16 h	24 h	48 h	72 h	96 h	120 h	Confirmation
Listeria	soil	4 C	nt	6/6	6/6	6/6	6/6	3/3	MOX agar; blackened agar around colony
		10 C	nt	6/6	6/6	4/6	2/6	0/3	
	no soil	4 C	nt	6/6	5/6	4/6	1/6	1/3	
		10 C	nt	5/6	1/6	2/6	0/6	0/3	
Salmonella	soil	4 C	6/6	6/6	6/6	6/6	6/6	5/6	XLT4 agar; clear colony with black center
		10 C	6/6	6/6	6/6	6/6	6/6	2/6	
	no soil	4 C	2/6	3/6	1/6	1/6	1/6	0/6	
		10 C	0/6	0/6	0/6	0/6	0/6	0/6	
Yersinia	soil	4 C	nt	7/9	2/9	0/9	2/9	0/3	*Yersinia* selective agar; red center
		10 C	nt	2/9	0/9	0/9	0/9	0/3	
	no soil	4 C	nt	0/9	1/9	0/9	2/9	0/3	
		10 C	nt	0/9	0/9	0/9	0/9	3/3	

nt = not tested

Figure 1. Petri dish containing stainless steel coupons.

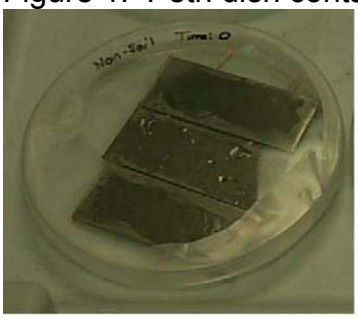
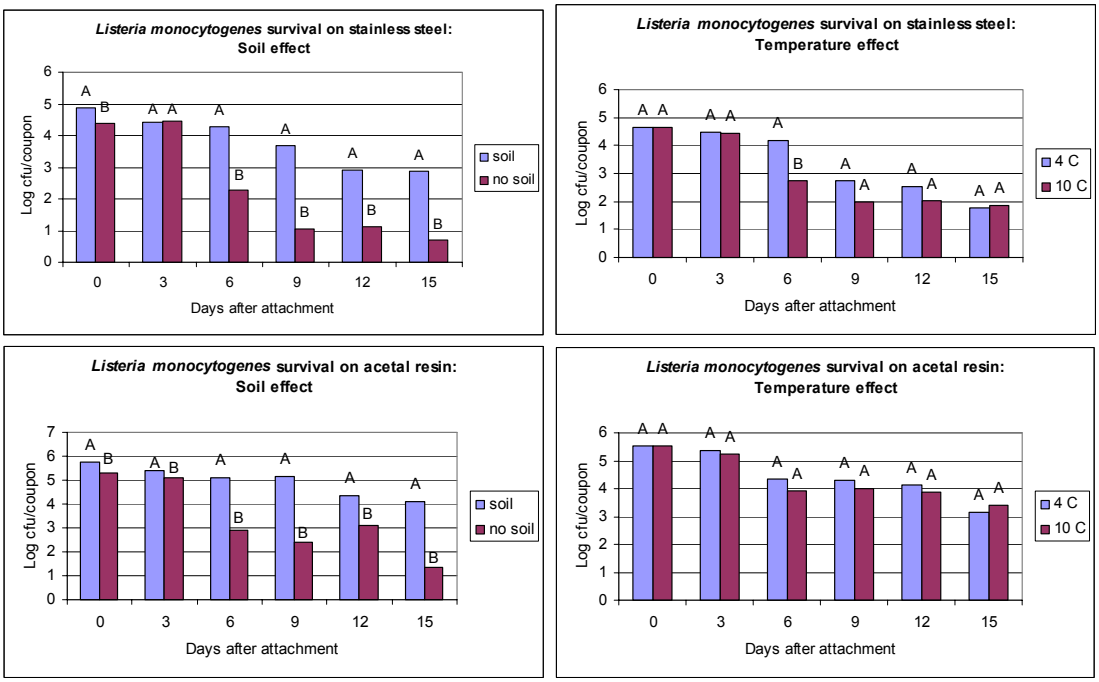
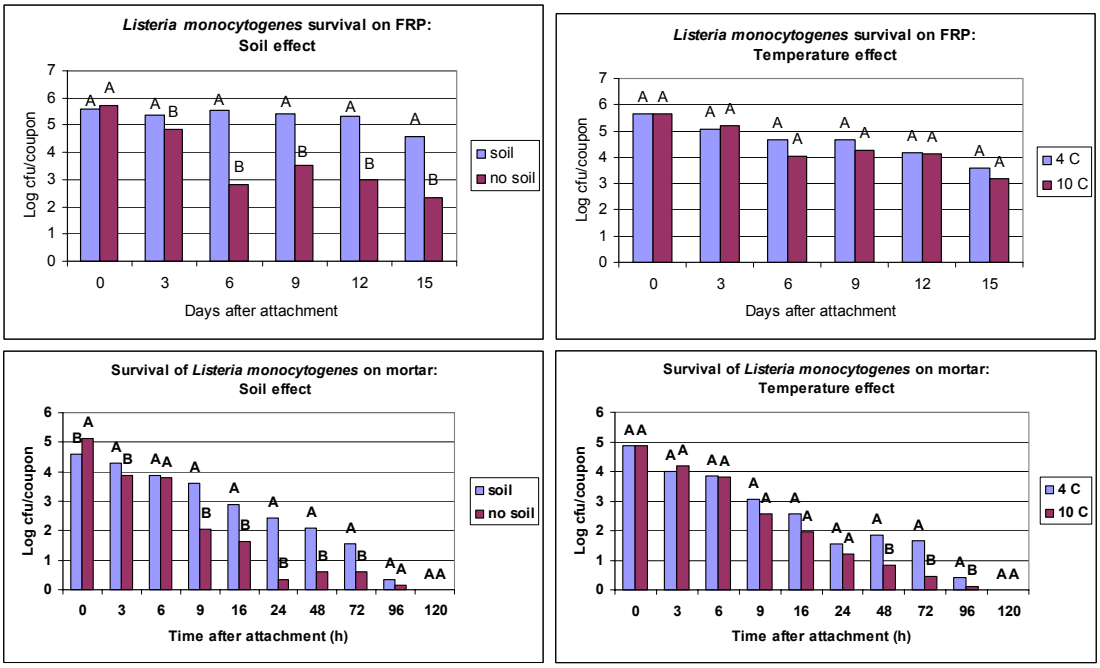


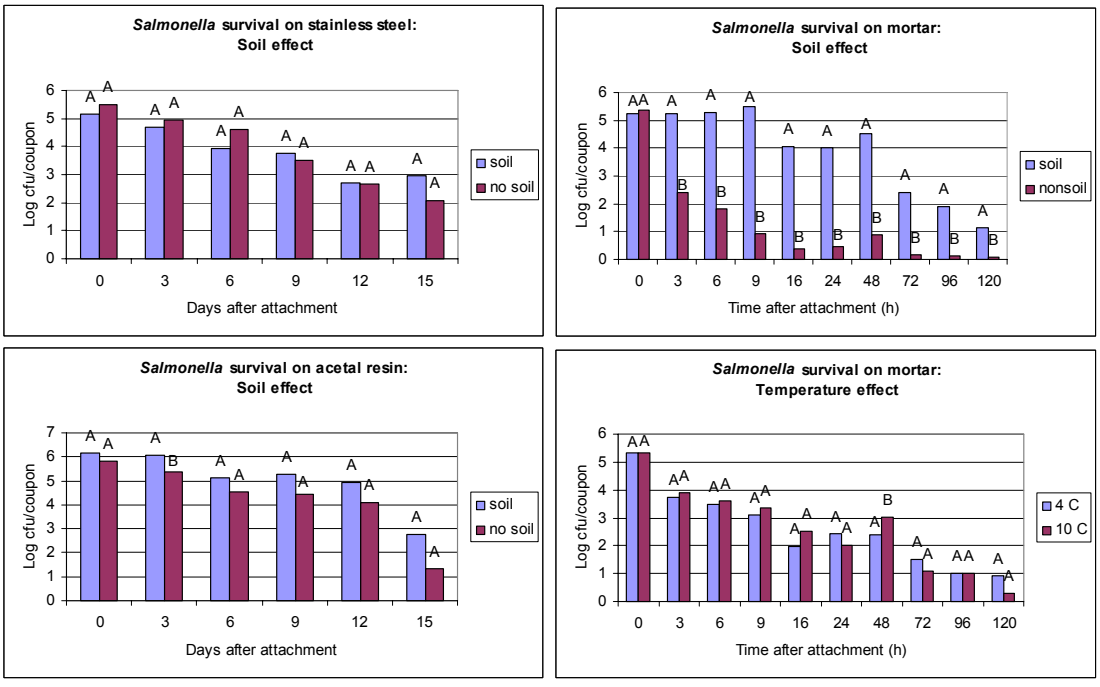
Figure 2. *Listeria monocytogenes*: Influence of soil and temperature on four surfaces.*

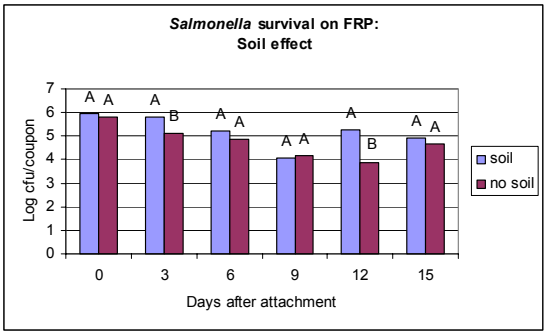




*At a particular time period, different letters above the pair of bars indicate a significant difference ($P \leq 0.05$) between the two means by Fisher's student *t*-test.

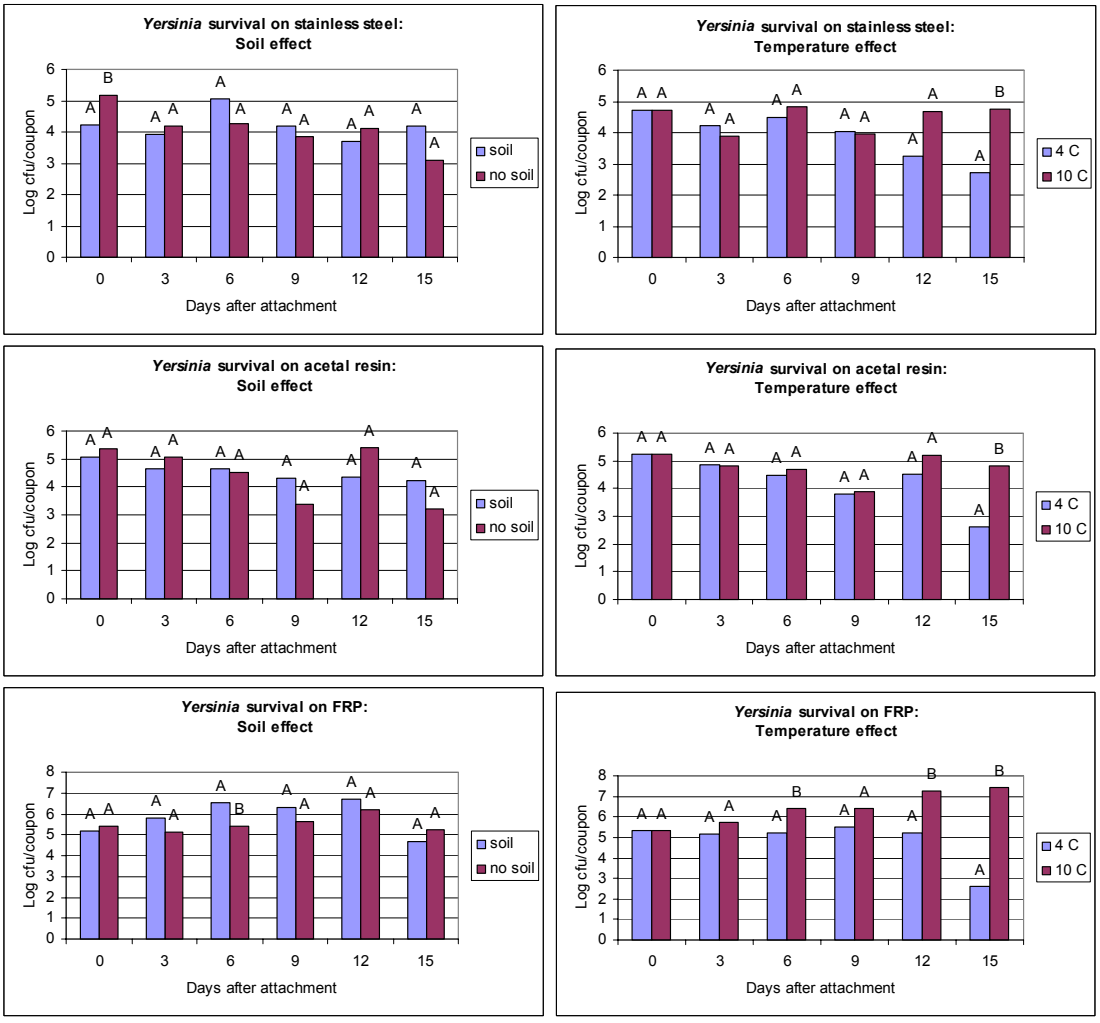
Figure 3. *Salmonella*: Influence of soil and temperature on four surfaces.*

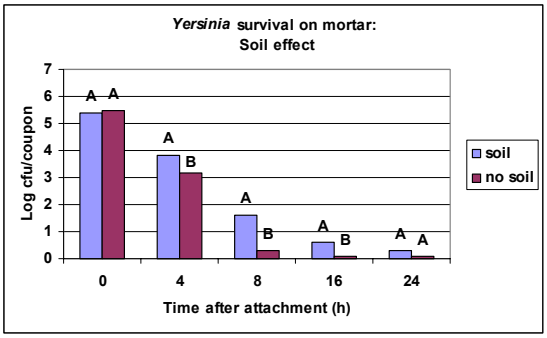


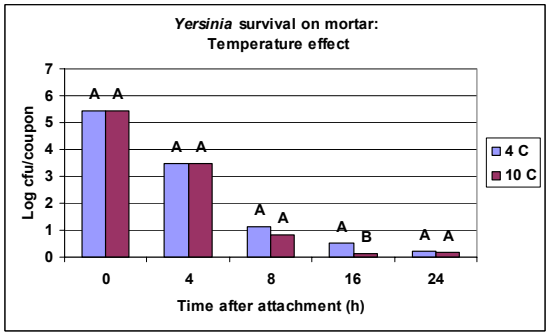


*At a particular time period, different letters above the pair of bars indicate a significant difference ($P \leq 0.05$) between the two means by Fisher's student *t*-test.

Figure 4. *Yersinia enterocolitica*: Influence of soil and temperature on four surfaces.*







*At a particular time period, different letters above the pair of bars indicate a significant difference ($P \leq 0.05$) between the two means by Fisher's student *t*-test.